Interaction of the Betapapillomavirus E2 Tethering Protein with Mitotic Chromosomes[▽]

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During persistent papillomavirus infection, the viral E2 protein tethers the viral genome to the host cell chromosomes, ensuring maintenance and segregation of the viral genome during cell division. However, E2 proteins from different papillomaviruses interact with distinct chromosomal regions and targets. The tethering mechanism has been best characterized for bovine papillomavirus type 1 (BPV1), where the E2 protein tethers the viral genome to mitotic chromosomes in complex with the cellular bromodomain protein, Brd4. In contrast, the betapapillomavirus human papillomavirus type 8 (HPV8) E2 protein binds to the repeated ribosomal DNA genes that are found on the short arm of human acrocentric chromosomes. In this study, we show that a short 16-amino-acid peptide from the hinge region and the C-terminal DNA binding domain of HPV8 E2 are necessary and sufficient for interaction with mitotic chromosomes. This 16-amino-acid region contains an RXXS motif that is highly conserved among betapapillomaviruses, and both arginine 250 and serine 253 residues within this motif are required for mitotic chromosome binding. The HPV8 E2 proteins are highly phosphorylated, and serine 253 is a site of phosphorylation. The HPV8 E2 chromosome binding sequence also has sequence similarity with chromosome binding regions in the gammaherpesvirus EBNA and LANA tethering proteins.

The life cycle of papillomaviruses is coupled to the epithelial differentiation of the host keratinocytes. Infection is initiated when viruses enter the basal cells of the epithelium through microabrasions or wounds. Upon infection, the viral genome replicates to a low copy number and is maintained as an extrachromosomal element that replicates in synchrony with the host cellular DNA. For some papillomaviruses, the viral E2 protein maintains and partitions the viral genomes by tethering them to the host chromosomes (3, 19, 26, 52). This mechanism ensures that the virus retains its genome within the nucleus of the infected cell and partitions its DNA to daughter cells after each cell division. This process helps maintain a persistent infection of the host; the dividing basal cells provide a reservoir of infected cells that migrate up to replenish the overlying, virus-producing, differentiated layers. In the productive phase of infection, genome amplification, synthesis of the capsid proteins. and assembly of virions occur in the differentiated upper layers of the epithelium where virus-containing cells are shed.

Human papillomavirus type 8 (HPV8) and type 5 (HPV5) belong to the *Betapapillomavirus* genus. They infect the cutaneous epithelium of humans and cause asymptomatic infections in healthy individuals (18). However, in individuals with epidermodysplasia verruciformis, a rare, inherited immune disorder, infection results in flat wart-like papules on the skin, which become cancerous after decades of infection (13). The betapapillomaviruses have also been implicated in nonmelanoma skin cancers (11).

The HPV E2 protein is multifunctional; it is involved in initiating viral DNA replication and regulating viral transcription, in addition to maintaining the genome as an extrachromosomal replicating element. The full-length E2 protein consists of an N-terminal domain (about 200 amino acids) linked by a flexible hinge region to a C-terminal DNA binding and dimerization domain (approximately 100 amino acids) (33). The E2 protein regulates viral transcription by binding to a 12-bp palindromic sequence with a consensus motif of $ACCN_6GGT$ that is present in the long control region of the viral genome (1). The N-terminal domain is important for the transcriptional regulation function of E2, interaction with the E1 replication protein, as well as interaction with many cellular proteins that are required for the transcriptional regulation of viral genes (38, 45, 48). In bovine papillomavirus type 1 (BPV1) E2, the N-terminal domain associates with mitotic chromosomes in complex with the Brd4 protein (4, 35, 59).

A study in our laboratory analyzed the mitotic binding phenotype of the E2 proteins from 13 different papillomaviruses belonging to six different genera (39). Most of the E2 proteins associated with mitotic chromosomes, but it was observed that the mitotic binding pattern varied among the E2 proteins from different genera. BPV1 E2 was detected as small speckles over the arms of all mitotic chromosomes. In contrast, the HPV8 E2 protein showed a distinct pattern of large foci bound to the pericentromeric regions of chromosomes (39). Our laboratory has shown that the HPV8 E2 protein associates with the ribosomal DNA (rDNA) loci present on the short arms of human acrocentric chromosomes and colocalizes with upstream binding factor (UBF) (44). UBF is a transcription factor required for rDNA transcription by RNA polymerase I (21); it remains bound to chromosomes during mitosis even when transcription is silenced (40, 46). Studies in our laboratory have shown that the domains required for mitotic chromosomal association of HPV8 E2 are different from those required for BPV1 E2 binding; the hinge and C-terminal domain are sufficient and

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essential for chromosomal association (44). Unlike BPV1 E2, the HPV8 N-terminal transactivation domain is not required for binding. Substitution of residues R37 and I73 in the transactivation domain abrogates Brd4 binding and mitotic chromosome binding of BPV1 E2, but an identical mutation does not abrogate chromosome association of HPV 8 E2 (44).

Other persistent DNA viruses have developed a similar strategy for genome maintenance and partitioning to enable them to persist in the host. Gammaherpesviruses Kaposi's sarcomaassociated herpesvirus and Epstein-Barr virus encode DNA binding proteins latency-associated nuclear antigen (LANA) and Epstein-Barr virus nuclear antigen 1 (EBNA1), respectively, which bind to specific sites in the viral DNA and tether the genome to host chromosomes (reviewed in reference 30). In addition to sharing the common function of genome tethering, these proteins also play an important role in viral genome replication and transcriptional regulation (14, 27). Structurally, all three proteins form, or are predicted to form, a similar dimeric beta-barrel C-terminal DNA binding structure, despite having no sequence homology (16; reviewed in reference 9). Short peptide sequences have been identified in LANA and EBNA1 that mediate binding to mitotic chromosomes (2, 6, 23, 29, 57).

In this study, we have defined specific residues within the HPV8 E2 hinge region that mediate the characteristic HPV8 E2 mitotic chromosomal binding pattern. We show that a highly conserved motif, RXXS, is required for this association and that the serine within this motif is phosphorylated in vivo. Furthermore, this sequence has similarities to the chromosome binding regions of the herpesvirus EBNA1 and LANA proteins.

MATERIALS AND METHODS

Plasmids. DNA fragments encoding domains or subdomain regions of the HPV8 and HPV5 E2 proteins were amplified using PCR primers with appropriate restriction sites. When appropriate, sequences were cloned into an engineered ClaI site located upstream from the C-terminal DNA binding domains, as outlined in Fig. 2A and 3B. Cloning details will be provided upon request. All E2 proteins were expressed with an N-terminal FLAG epitope tag from an inducible metallothionein promoter in the pMEP-4 expression vector as described previously (42). The C-terminal domain was expressed either with a FLAG tag fused upstream of residue 404 (C) or with an additional calmodulin binding protein and streptavidin binding protein tag cloned between the FLAG tag and C-terminal DNA binding domain (CTD) (Tag-C) as described previously (44).

Mutagenesis. Two sets of E2 point mutations were generated. One set was designed in the background of a 40-amino-acid region from the HPV8 E2 hinge (residues 216 to 255) fused to the CTD. The second set was designed in the background of a 16-amino-acid region from the hinge (residues 240 to 255) fused to the CTD. DNA fragments with the respective amino acid substitutions were chemically synthesized and cloned in frame with the CTD. All E2 plasmids were sequenced to confirm the mutations.

Establishment of stable pMEP-E2 cell lines. Stable cell lines derived from African green monkey CV-1 cells and human C33A cells were generated by transfection of the pMEP-E2 plasmids with Fugene 6 (Roche) and selection with 200 μg/ml or 60 μg/ml hygromycin B (Roche), respectively. After 2 weeks, drug-resistant colonies were pooled, and cultures were expanded.

Immunoblotting. Cellular proteins were extracted in 2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 6.8), 10% glycerol, and Complete protease inhibitor (Roche). Protein concentration was determined using the BCA (bicinchoninic acid) protein assay kit (Pierce). For each sample, 12 μg of protein was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto Immobilon-P membranes (Millipore). E2 proteins were detected with M2 anti-FLAG monoclonal antibody (Sigma) and then with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce). Immune complexes were detected on the membrane with the chemiluminescent reagent SuperSignal West Dura (Pierce).

Indirect immunofluorescence. CV-1 cells containing pMEP plasmids were grown on Superfrost Plus slides. For mitotic synchronization, cells were blocked in S phase with 2 mM thymidine for 14 to 16 h. The thymidine was removed, and the cells were cultured for an additional 9 h with E2 expression induced by the addition of 3 μM CdSO$_4$ during the last 4 h. The cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 15 min at room temperature. The E2-derived proteins were detected with monoclonal anti-FLAG M2 antibody (1:500; Sigma), UBF was detected with rabbit anti-UBF antiserum (1:100; Santa Cruz Biotechnologies, Inc.), and nuclear speckles were detected with anti-SC35 (1:500; Sigma). Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories. Slides were mounted in Vectashield containing 1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole). Images were collected with a Leica TCS-SP5 laser scanning confocal imaging system.

In vivo phosphorylation assay. E2 protein expression was induced for 3 h with 3 μM CdSO$_4$ in medium containing 0.42 mCi/ml [^{32}P]orthophosphate or a combination of 0.2 mCi/ml [^{35}S]methionine and [^{35}S]cysteine. Cell extracts were prepared in modified radioimmunoprecipitation assay (RIPA) buffer (20 mM HEPES [pH 7.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate, 0.1% SDS) containing Complete protease inhibitor cocktail (Roche) and PhosSTOP (Roche). E2-derived proteins were immunoprecipitated using M2 anti-FLAG antibody beads (Sigma). Immune complexes were washed five times with RIPA buffer, and E2 proteins were eluted in 2% SDS-PAGE sample buffer and separated by SDS-PAGE. Gels were fixed, dried, and autoradiographed. Quantitation was performed using a Typhoon PhosphorImager and ImageQuant software (GE).

RESULTS

Regions in the hinge of the HPV8 E2 protein required for mitotic chromosomal interaction. Both the hinge and the C-terminal DNA binding and dimerization domain are essential and sufficient for HPV8 E2 mitotic chromosome binding (44). The hinge region of the betapapillomavirus E2 proteins is unusual in composition compared to the E2 proteins from other genera of papillomaviruses. The hinge is not well conserved with other papillomaviruses, but within the *Betapapillomavirus* genus, the hinge regions have similar lengths and sequence compositions. The human betapapillomavirus (betaHPV) E2 hinge regions are longer than those of most papillomavirus E2 proteins and are rich in RG and RS dipeptide motifs. The amino acid sequence of the HPV8 E2 protein hinge is shown in Fig. 1. The E2 hinge regions are thought to be unstructured, and this has been experimentally shown for HPV16 E2 (15). The regions of the EBV EBNA1 protein required for mitotic chromosome binding contain AT hooks (47). The amino acid sequence of the HPV8 E2 hinge region has stretches of sequence with similarities to AT hook motifs, but there is no exact match with the AT hook consensus motif (PRGRP). In this study, we have defined the sequences from the hinge that are essential for the characteristic HPV8 E2 foci on mitotic chromosomes.

E2 proteins were expressed in CV-1 cells to identify regions within the E2 hinge required for mitotic chromosome association. The localization of each protein was analyzed by indirect immunofluorescence using an antibody against an N-terminal FLAG epitope tag. We have previously shown that HPV8 E2 is observed in distinct foci in the pericentromeric regions of mitotic chromosomes in both African green monkey CV-1 cells and human C33A cells (39). We have mapped this localization in human C33A cells to the rDNA loci on the short arms of acrocentric chromosomes (44). In CV-1 cells, approximately three UBF foci are observed, and the HPV8 E2 protein associates with these regions (data not shown). However, there are

206

S P P G Q A D T D T A A K T P T T S A D S T S R Q Q R S P A K Q P Q Q T E T K G R R Y G R R P S S R

T R P Q K E Q R R S R S R H R T R S R S R S L S R V R A V G S T T V S R S R S S S L T K A V R P R S

R S R S R G R A T A T S R R R A G R G S P R R R R S T S R S P S T N T F K R S Q R G G G R R G R G R

G S R G R R E R S S S T S P T P T K R S R G E S S R L R G V S P S E V G R S V Q S V S A K H T G

403

FIG. 1. Amino acid sequence of the HPV8 E2 protein hinge. Amino acids 206 to 403 from the hinge region of HPV8 E2 are shown. SR and RS dipeptides are shown in red, and the 16-amino-acid chromosome binding region mapped in this study is underlined.

additional pericentromeric foci in CV-1 cells that do not overlap with UBF, and we are currently characterizing these additional pericentromeric regions. All of the HPV8 E2 proteins used in this study associated with mitotic chromosomes in a similar pattern; they completely colocalized with UBF and rDNA foci in C33 cells, and they associated with both UBF-positive and -negative foci in CV-1 cells.

A series of E2 proteins were expressed; these E2 proteins contained an intact C-terminal domain (residues 404 to 498) fused to various sequence segments from the hinge region (Fig. 2A and B). As shown in Fig. 2C and Table 1, E2 proteins were examined for their ability to bind to mitotic chromosomes in the distinct foci characteristic of HPV8 (39). The N-terminal region of the hinge was truncated at amino acid positions 216, 240, 255, 286, and 313. Deletion up to position 240 had no effect on chromosomal association, but further deletion abrogated the characteristic HPV8 E2 binding foci. Deletions were also generated to remove C-terminal portions of the hinge with endpoints at residues 385, 366, 338, 312, 290, 272, 255, and 240. In this case, deletion up to residue 255 had no effect on chromosome association, but further deletion to residue 240 eliminated binding. Correspondingly, a small region from the hinge consisting of residues 240 to 255 fused to the C-terminal domain (240-255-C) was able to associate with mitotic chromosomes (Fig. 2A and C). Furthermore, the 240-255-C mitotic foci colocalized with upstream binding factor in a fashion similar to that of the full-length E2 protein (data not shown). Thus, a 16-amino-acid region, when fused to the CTD, can mediate mitotic chromosomal binding.

The HPV8 E2 proteins localize to nuclear speckles, but this does not correlate with mitotic chromosome binding. Proteins from the SR family of splicing factors contain multiple RS motifs and localize to nuclear speckles, where they are intimately involved in transcription and splicing (reviewed in reference 28). The E2 protein from the betapapillomavirus HPV5 localizes to these speckles (25), and this localization is dependent on the hinge region. We found that the HPV8 E2 protein is also localized to the nuclear speckles in interphase cells, as shown by colocalization with SC35, a marker for nuclear speckles (Fig. 3). To determine which domains of the HPV8 E2 protein are required for this colocalization, FLAG-tagged E2 proteins containing various combinations of the N-terminal (N), hinge (H), and C-terminal domain (C) were expressed and analyzed for colocalization with the SC35 protein. As shown in Fig. 3A, the hinge region was required for this localization, but it was not sufficient. The hinge region alone (H) did not associate with nuclear speckles, and a combination of

the N-terminal domain and hinge region (NH) resulted in only weak colocalization with SC35. In fact, the absence of the C-terminal domain resulted in nucleolar localization of these E2 proteins. However, a protein consisting only of the hinge and C-terminal domain (HC) gave strong nuclear speckle localization. Therefore, a function of the C-terminal domain, such as DNA binding or dimerization, might be important to augment this localization. Notably, the same two domains are required for mitotic chromosome association.

The E2 proteins with truncations in the hinge region were also analyzed for their ability to localize to nuclear speckles to determine whether this was dependent on the multiple SR dipeptide motifs and to determine whether this association correlated with mitotic chromosome localization. The sequence of amino acids that was found to be important for mitotic chromosomal association (residues 240 to 255) contains one SR dipeptide motif (Fig. 1). Deletion of the C-terminal half of the hinge in the background of the full-length E2 protein resulted in only a minimal decrease in nuclear speckle localization (N-206-312-C) (Fig. 3B). However, a dramatic decrease was observed when the N-terminal half of the hinge was deleted (N-313-403-C and 313-403-C). Progressive truncation of the N-terminal half of the hinge resulted in decreased nuclear speckle binding. In the absence of the N-terminal transactivation domain, this resulted in a progressive increase in nucleolar localization. However, neither half of the hinge was sufficient to localize the proteins to nuclear speckles, despite the presence of multiple SR sequences. We postulate that either a minimal number of SR motifs is required for this localization, that specific sequences from both halves of the hinge are required, or that the deletion endpoint at residues 312/313 interrupts a crucial element. In interphase, the minimal chromosomal binding E2 protein, 240-255-C, was localized throughout the nucleus and nucleolus in a diffuse staining pattern and did not localize specifically to nuclear speckles. Therefore, nuclear speckle association does not correlate with mitotic chromosome binding.

The chromosome binding region is functional in other betapapillomavirus E2 proteins. To confirm that the pattern of chromosome binding seen with HPV8 E2 is common to other betapapillomavirus E2 proteins, we examined the association of HPV5 E2 with mitotic chromosomes. As shown in Fig. 4, the mitotic localization of HPV5 E2 was similar to HPV8 E2 with E2 detected as large distinct foci on the chromosomes. These foci also overlapped with the RNA polymerase I transcription factor, UBF (data not shown). To show that the chromosome binding region mapped in HPV8 E2 was also functional in HPV5 E2, we expressed an HPV5 E2 protein carrying a 40-
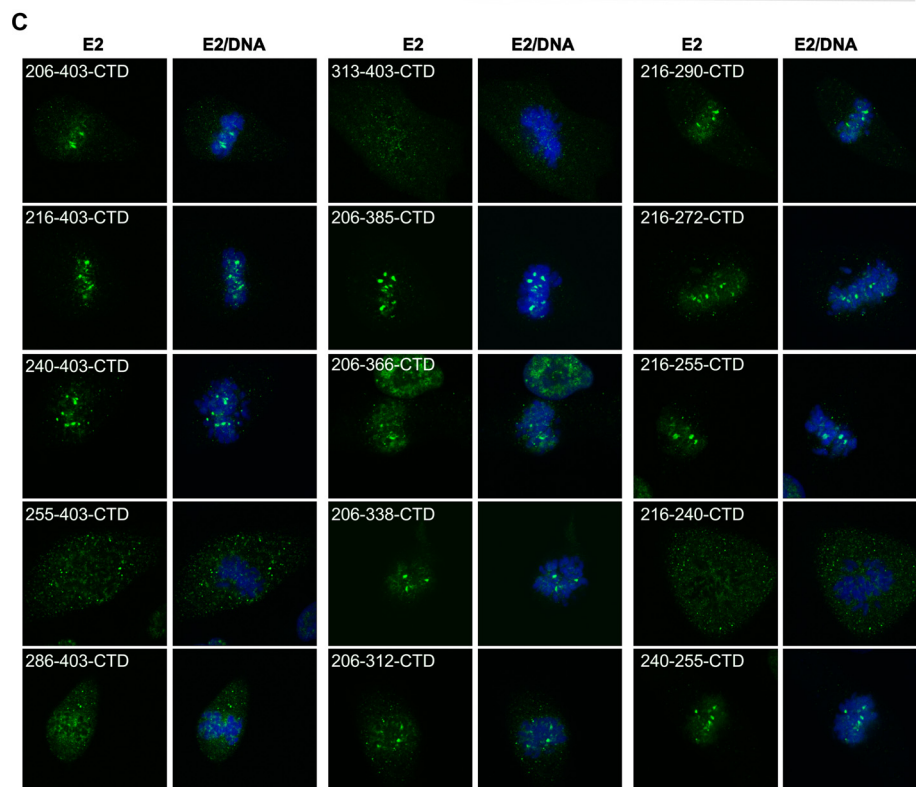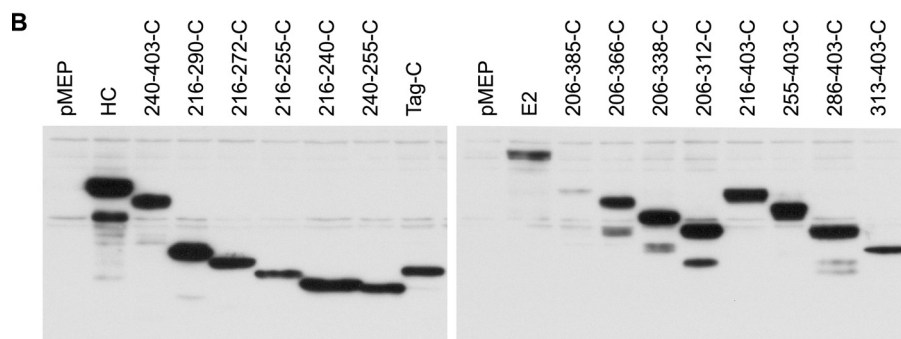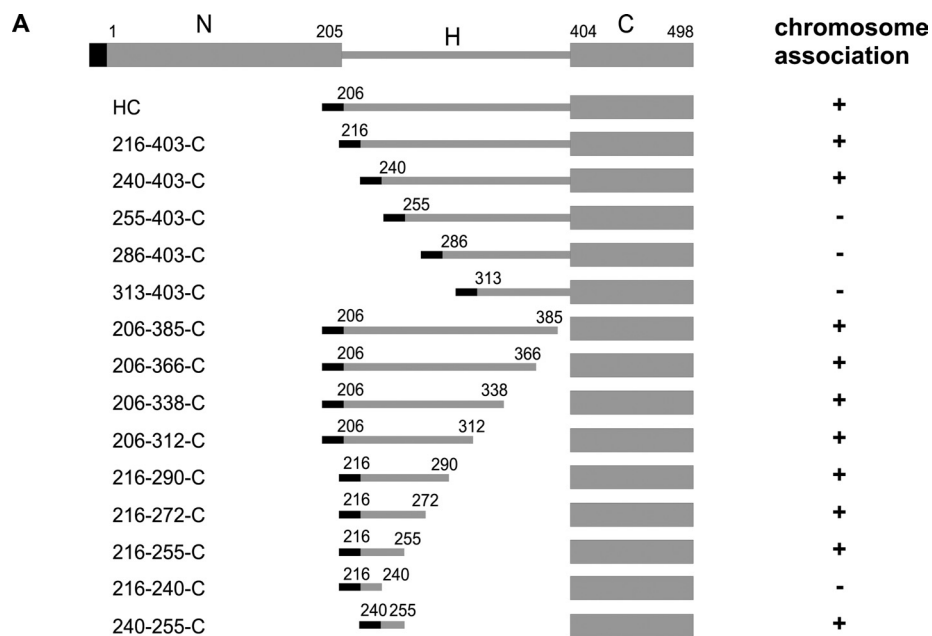
TABLE 1. E2-expressing mitotic cells showing the focus binding
pattern shown in Fig. 2C

E2 protein	No. of mitotic cells examined	% Cells expressing E2	% E2-positive cells with E2 foci on mitotic chromosomes
E2	41	46.3	89.5
E2HC	40	55.0	95.5
216-404-C	29	75.9	100
240-404-C	41	56.1	82.6
255-404-C	54	38.9	0
286-404-C	42	40.5	0
313-404-C	30	36.7	0
216-240-C	35	34.3	0
216-255-C	35	82.9	93.1
216-272-C	27	85.2	73.9
216-290-C	40	72.5	100
240-255-C	37	43.2	81.3
207-312-C	36	69.4	100
207-338-C	34	79.4	100
207-366-C	44	75.0	100
207-385-C	34	88.2	100

amino-acid region similar to the mapped chromosome binding region of HPV8 (residues 216 to 255) fused to the C-terminal DNA binding domain. The HPV5 216-255 C protein was also observed to associate with mitotic chromosomes (Fig. 4) and to colocalize with UBF (data not shown). We also carried out experiments in which the C-terminal domains and 40-residue hinge chromosome binding regions from each E2 protein were exchanged (Fig. 4). The pattern of mitotic binding was the same for each of the E2 fusion proteins (Fig. 4). Hence, from this study, we can conclude that the mitotic binding pattern observed with the HPV8 E2 protein is also observed in other betapapillomavirus E2 proteins and involves a similar region of the hinge region along with the DNA binding domain. Figure 4D shows a comparison of the regions from residues 216 to 255 of HPV5 and HPV8. Notably, the smaller 16-amino-acid chromosome binding region (region from residues 240 to 255; underlined in HPV8) is the most homologous region within the larger 40-amino-acid peptide.

The chromosome binding regions of the betapapillomaviruses are highly conserved. Elements sufficient for mitotic binding of HPV8 and HPV5 E2 map to the same hinge region of the proteins. To assess whether this region is also conserved among the five different species of betapapillomaviruses, the predicted E2 sequences from 29 different betapapillomaviruses, belonging to species 1, 2, 3, 4, and 5, were analyzed using the Clustal W alignment program (Fig. 5). The alignment showed that the 16-amino-acid chromosome binding region contains a small stretch of the most highly conserved residues in the E2 hinges of all betapapillomaviruses. Hence, we have
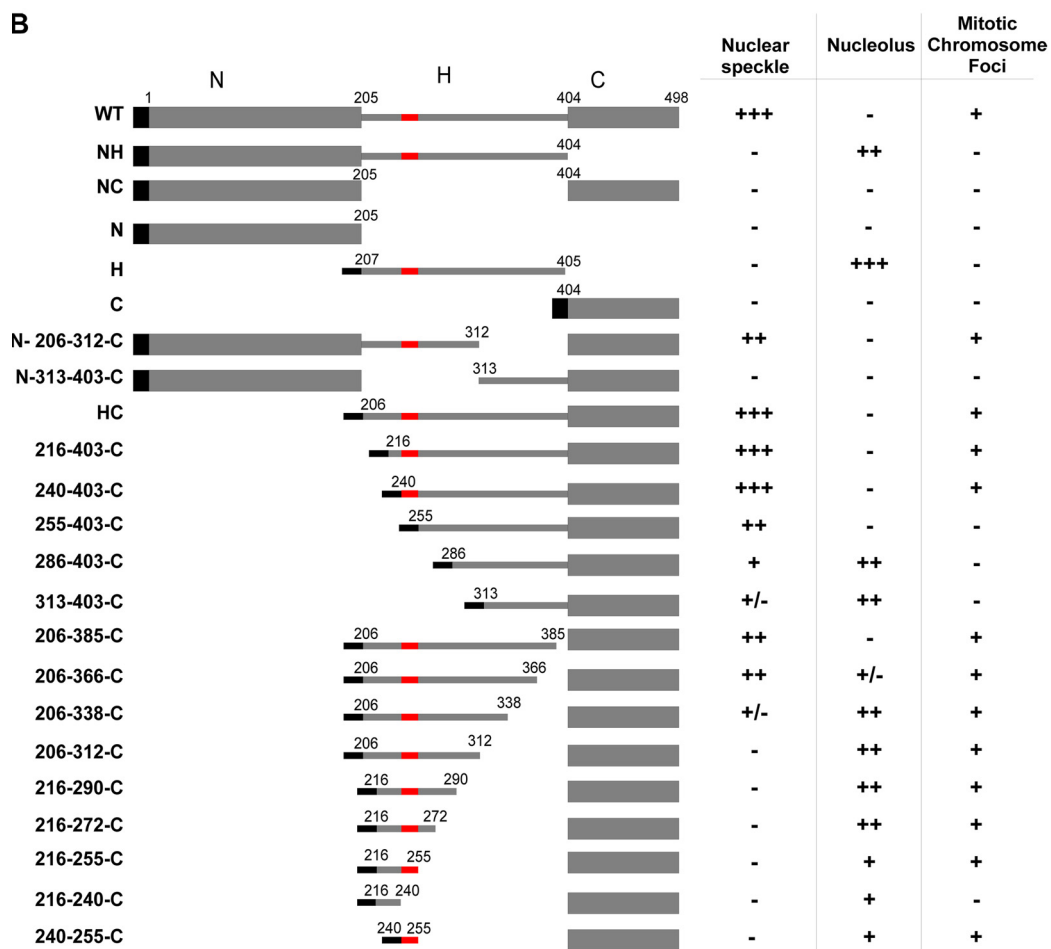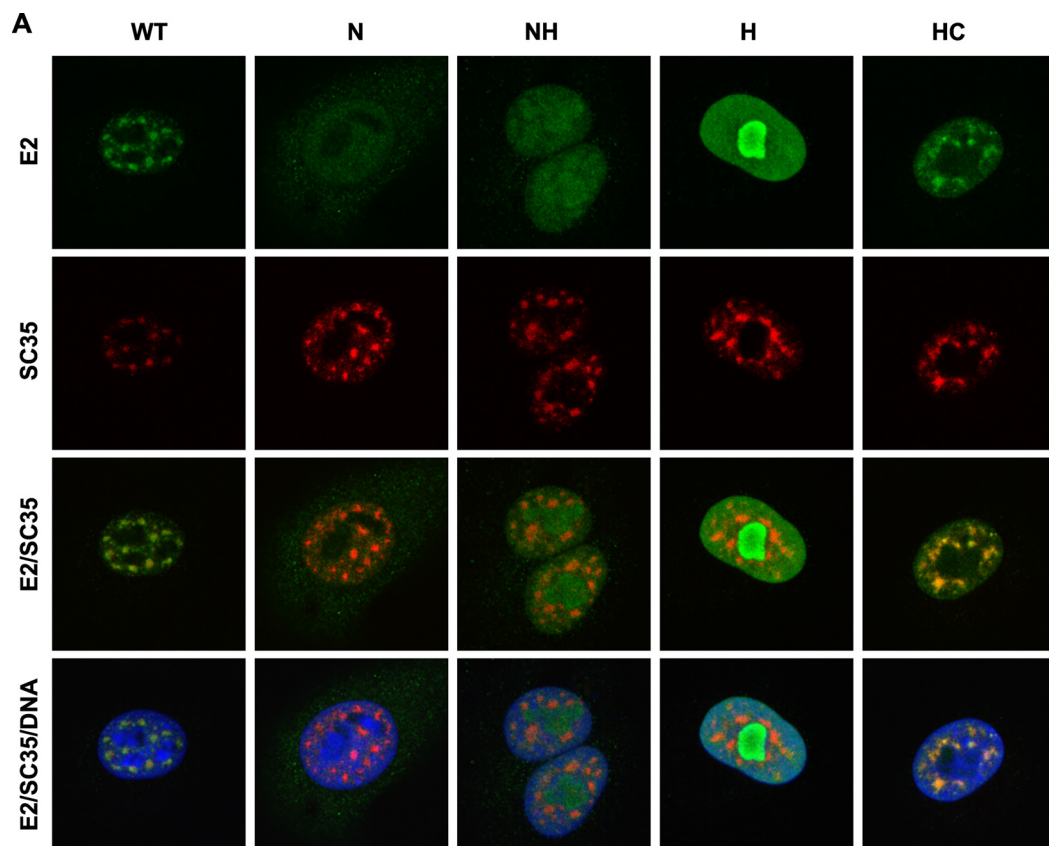
mapped the region of E2 required for mitotic chromosomal association to a short peptide that is highly conserved among all beta-HPVs. The fact that this region has been conserved throughout evolution in an otherwise divergent region of the E2 protein suggests that it has a similar function in all beta-HPV E2 proteins.

An RXXS motif in the hinge is crucial for mitotic chromosome binding. To further map specific residues in the hinge important for mitotic chromosomal interaction of the HPV8 E2 protein, we generated specific amino acid substitutions. These studies were initiated at the point when we had mapped the chromosomal binding region to the 40 residues between amino acids 216 and 255 of the hinge, so point mutations were generated in a background of these 40 residues fused to the C-terminal domain. This region contains a few residues that are highly conserved among all betapapillomavirus E2 proteins and also carries a number of putative consensus sites for different modification enzymes, such as CK2, Aurora kinases, and protein arginine methyltransferases. Key residues were substituted with alanine to eliminate sites of possible posttranslational modifications, such as arginine methylation (R250/251) and serine/threonine phosphorylation (T219, SP233/234, and SS253/254), and to change conserved residues (P220A, D225, QQ239/240, Y248, GG245/249, and P252). The sequences of these mutations and levels of expression of the mutated E2 proteins are shown in Fig. 6A and B, respectively.

The mutated 216-255-C E2 proteins were tested for their ability to bind mitotic chromosomes by immunofluorescence (Fig. 6C and Table 2). Only two of the mutated E2 proteins, RR250/251AA and SS253/254AA, showed a loss of the chromosome binding phenotype. Neither protein was observed in the characteristic, distinct HPV8 chromosomal foci. All of the other mutated E2 proteins exhibited the wild-type pattern of mitotic localization. Since the full-length E2 protein colocalizes with UBF on mitotic chromosomes (44), these mutated E2 proteins were also tested for their colocalization with UBF. All mutated E2 proteins, except RR250/251AA and SS253/254AA showed overlapping localization with UBF on mitotic chromosomes (data not shown). Thus, the region between positions 250 and 254 in the HPV8 E2 hinge region is important for HPV8 mitotic chromosomal binding.

Further examination of the RR250/251AA and SS253/254AA amino acid sequences determined that both mutations disrupted two overlapping "RXXS" kinase motifs. This RXXS motif is a common consensus for several different kinases, such as the Aurora kinases, protein kinases A and C, calmodulin-dependent kinases, and the ROCK kinases (37, 55, 58). Furthermore, these four residues map to the region of 16 amino acids (residues 240 to 255) identified in parallel by deletion

FIG. 2. Chromosome binding phenotypes of E2 proteins with deletions in the hinge. (A) Schematic representations of E2 proteins truncated in the hinge region and their chromosome binding phenotypes. Truncated E2 proteins were generated as fusion proteins with the indicated hinge (H) sequence fused to the CTD (C). The FLAG epitope is shown at the N terminus (N) as a black box. Chromosome association is shown to the right of the schematic representations of E2 proteins as follows: +, chromosome association; −, no chromosome association. (B) Immunoblot analysis of the E2 proteins, as detected with an anti-FLAG antibody. (C) Immunolocalization of truncated HPV8 E2 proteins in mitotic CV-1 cells. E2 proteins, as detected with a fluorescein isothiocyanate (FITC)-labeled antibody, are shown in green. Cellular DNA was stained with DAPI (blue). The percentage of mitotic cells expressing E2 protein and the percentage of positive E2-expressing mitotic cells showing the focus binding pattern are shown in Table 1.

A

	WT	N	NH	H	HC
E2					
SC35					
E2/SC35					
E2/SC35/DNA					

B

		Nuclear speckle	Nucleolus	Mitotic Chromosome Foci
WT	N 1 ... 205 H ... 404 C 498	+++	-	+
NH	404	-	++	-
NC	205 ... 404	-	-	-
N	205	-	-	-
H	207 ... 405	-	+++	-
C	404	-	-	-
N- 206-312-C	312	++	-	+
N-313-403-C	313	-	-	-
HC	206	+++	-	+
216-403-C	216	+++	-	+
240-403-C	240	+++	-	+
255-403-C	255	++	-	-
286-403-C	286	+	++	-
313-403-C	313	+/-	++	-
206-385-C	206 ... 385	++	-	+
206-366-C	206 ... 366	++	+/-	+
206-338-C	206 ... 338	+/-	++	+
206-312-C	206 ... 312	-	++	+
216-290-C	216 ... 290	-	++	+
216-272-C	216 272	-	++	+
216-255-C	216 255	-	+	+
216-240-C	216 240	-	+	-
240-255-C	240 255	-	+	+

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analyses. To further analyze which of the two serine and arginine residues were actually contributing to the mitotic binding phenotype, we designed another set of mutations. In this case, they were generated in the background of the 16 amino acid residues from positions 240 to 255 fused to the C-terminal DNA binding domain (240-255-C). Residues arginine 250 and 251 and serine 253 and 254 were individually substituted with alanine residues. Each serine residue was also substituted with aspartic acid to mimic the effect of constitutive phosphorylation. To identify additional residues required for chromosomal association, the remaining E2 proteins in this set also contained substitutions of conserved residues that had not been previously mutated in this region (RR246/247, E242, and K244). Finally, a protein in which all five potential phosphorylation sites in the region from positions 240 to 255 were substituted with alanine (A5) was generated. The sequences of these mutations and the levels of expression of the mutated proteins are shown in Fig. 7A and B, respectively.

Figure 7C shows the intracellular mitotic localization of the mutated 240-255-C proteins. The results of this analysis revealed that arginine 250 and serine 253 were crucial for mitotic localization of the HPV8 E2 protein; E2 proteins with R250A and S253A, as well as with S253D, were deficient in mitotic binding function and did not form mitotic chromosomal foci (Fig. 7C and Table 3). These residues lie within the $R_{250}XXS_{253}$ motif. As mentioned above, this is a motif common to a number of protein kinases. However, the phosphormimetic E2 protein with S253D was not able to substitute for E2 with phosphorylated S253, as this protein was excluded from mitotic chromosomes. The overlapping $R_{251}XXS_{254}$ motif was not required, as R251A and S254A E2 proteins could bind mitotic chromosomes. Chromosome binding was also abolished in the A5 E2 protein, which had alanine substitutions in all potential phosphorylation sites within the region from positions 240 to 255. All other mutated E2 proteins exhibited a binding pattern similar to that of the wild-type protein. The E2 proteins that bound to mitotic chromosomes also colocalized with UBF (data not shown). Therefore, the motif $R_{250}XXS_{253}$ in the hinge region is essential for the mitotic binding function. Notably, arginine 250 and serine 253 and the consensus motif "RXXS" are completely conserved among all of the betapapillomaviruses examined (Fig. 5).

HPV8 E2 serine 253 is a phosphorylation site. To investigate the phosphorylation status of the E2 proteins, we expressed the E2 proteins in CV-1 cells in the presence of [^{32}P]orthophosphate or a combination of [^{35}S]methionine and [^{35}S]cysteine. E2 proteins were immunoprecipitated from equivalent counts of total protein and analyzed by gel electrophoresis, auto-

radiography, and phosphorimaging (Fig. 8). An initial study included the full-length E2 protein, the HC protein (206-403-C) and the series of mutated proteins shown in Fig. 6A in the 216-255-C background. Figure 8A shows that the wild-type, HC, and 216-255-C proteins were all labeled with ^{32}P, indicating that they were phosphorylated. Notably, the full-length E2 and HC proteins were highly phosphorylated, as determined by the ratio of ^{32}P to ^{35}S incorporation, compared to the smaller 216-255-C protein. However, all 216-255-C proteins containing the amino acid substitutions shown in Fig. 6A were also phosphorylated. There was a slight reduction in ^{32}P incorporation in the RR251/252AA and SS253/254AA proteins, but it was difficult to conclude whether this was due to the elimination of phosphorylation in one of the overlapping RXXS motifs because of the potential background phosphorylation due to the additional 12 potential sites of phosphorylation in the region from positions 216 to 255 (data not shown).

To further determine whether the $R_{250}XXS_{253}$ motif is phosphorylated, the experiment was repeated with the series of mutated proteins in the 240-255-C background. As shown in Fig. 8B, the 240-255-C protein was phosphorylated (and minimal, if any, phosphorylation was detected with C alone). Mutation of all five potential phosphorylation sites (A5) also eliminates detectable phosphorylation. Furthermore, both S253A and S253D proteins have no detectable phosphorylation, confirming that residue 253, in the $R_{250}XXS_{253}$ motif, is a phosphorylation site. The 240-255-C proteins with substitutions in residue 254 (S254A and S254D) were phosphorylated, so S254, in the overlapping $R_{251}XXS_{254}$ motif, is not a major phosphorylation site. However, substitution of either R250 or R251 resulted in a substantial decrease in phosphorylation. Therefore, the optimal motif for phosphorylation is RRXS. However, although the R251A 240-255-C protein showed greatly reduced phosphorylation, it was able to bind to mitotic chromosomes in the characteristic HPV8 E2 binding foci. Therefore, even although R251 and S253 are essential for association with mitotic chromosomes, phosphorylation of the $R_{250}XXS_{253}$ motif may not be absolutely required. Alternatively, the minimal phosphorylation observed may be sufficient for the observed chromosome binding. The in vivo labeling experiments were carried out in asynchronous cells, and if the observed phosphorylation is cell cycle regulated, the residual phosphorylation might be present at higher levels in mitotic cells.

DISCUSSION

The papillomavirus E2 proteins are pivotal to the viral life cycle. The E2 open reading frames of most papillomaviruses

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FIG. 3. The HPV8 E2 protein localizes to SR nuclear speckles. (A) Colocalization of E2 and SC35 in interphase cells. CV-1 cells expressing full-length E2 (wild type [WT]) or expressing the N-terminal domain (N), the N-terminal domain and hinge region (NH), hinge (H), or hinge and C-terminal domain (HC) E2 domains were assayed for E2 and SC35 localization by indirect immunofluorescence. The E2 protein, as detected by a FITC-labeled antibody, is shown in green; the SC35 protein, as detected by a Texas Red antibody, is shown in red; colocalization of these proteins appears as yellow in the merged images. Cellular DNA was stained with DAPI (blue). (B) Summary of the intracellular localization of the HPV8 E2 proteins. All E2 proteins used in this study are shown with their intranuclear localization indicated to the right. Nuclear speckles were determined by colocalization with SC35. −, no apparent colocalization with SC35; +/−, minimal colocalization with SC35; +, low colocalization with SC35; ++, moderate colocalization with SC35; and +++, high colocalization with SC35. Nucleolar localization was determined by colocalization with UBF. −, minimal nucleolar localization; +, equal detection in the nucleus and nucleolus; ++ and +++, moderate and large amounts, respectively, of protein accumulated in the nucleolus. The 16-amino-acid chromosome binding region mapped in this study is shown in red.
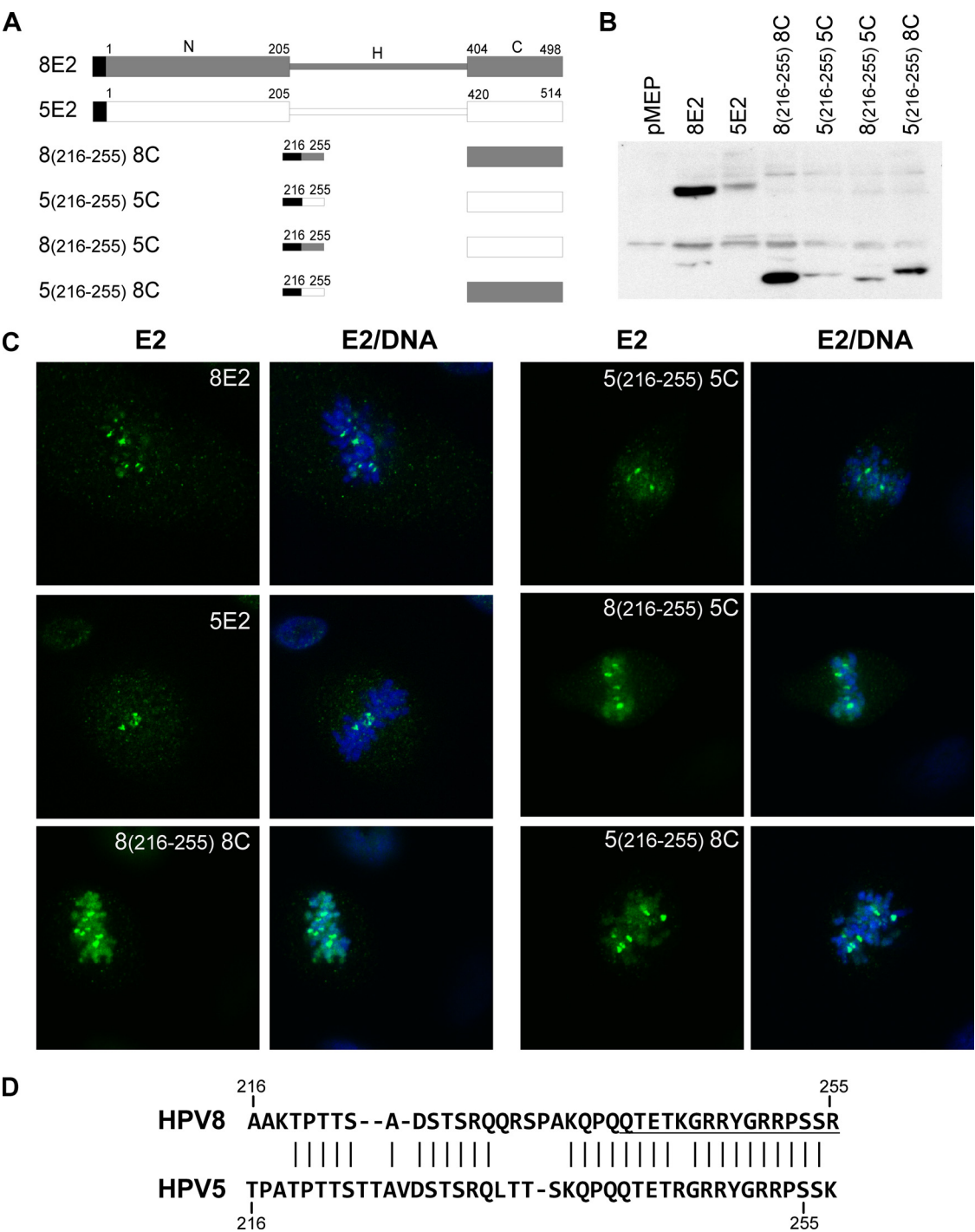
FIG. 4. HPV5 E2 protein shows a mitotic binding pattern similar to that of the HPV8 E2 protein. (A) Schematic representations of the HPV8 and HPV5 E3 fusion proteins. (B) Immunoblot analysis showing the expression levels of the full-length and fusion proteins of HPV8 and HPV5 E2. E2 was detected by using an anti-FLAG antibody. (C) Immunolocalization of HPV8 and HPV5 full-length and fusion E2 proteins in mitotic CV-1 cells. E2 proteins, as detected with a FITC-labeled antibody, are shown in green. Cellular DNA was stained with DAPI (blue). (D) Homology between the HPV8 and HPV5 hinge regions from residues 216 to 255. The 16-amino-acid chromosome binding region is underlined. Gaps introduced to maximize alignment are indicated by hyphens. The vertical lines between the HPV8 and HPV5 sequences indicate identical amino acids.

encode both a full-length E2 protein containing the transactivation domain, the hinge region, and the DNA binding and dimerization domain and shorter "repressor" proteins that contain only the C-terminal domain and hinge sequences. The full-length protein can both activate and repress transcription and initiates DNA replication in complex with the E1 protein. The shorter E2 proteins can antagonize these functions. For several papillomaviruses, the full-length E2 proteins link the viral genome to the host chromosomes to support maintenance, stability, and partitioning of the viral genome.
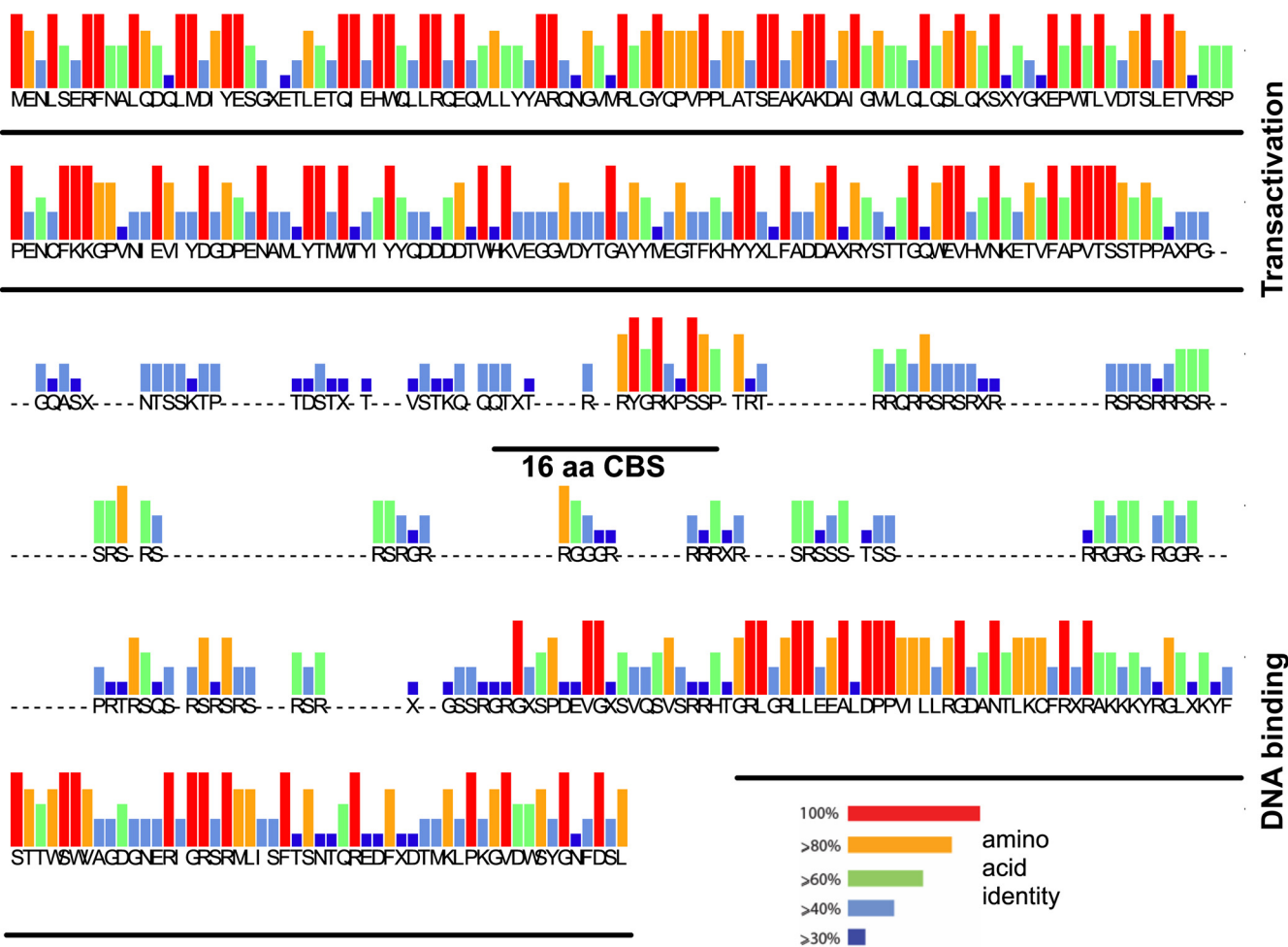
FIG. 5. Sequence analysis of the chromosome binding region. Clustal W alignment of E2 proteins from 29 papillomaviruses belonging to the five different species of the *Betapapillomavirus* genus. Residues tyrosine 248, arginine 250, and serine 253 are 100% conserved in these 29 sequences. The 16-amino-acid chromosome binding sequence (16-aa CBS) is underlined. The transactivation domain and DNA binding dimerization domain are indicated.

For BPV1, both the N-terminal transactivation domain and C-terminal DNA binding domain of E2 are required for efficient mitotic chromosome interaction and genome partitioning (3, 19, 52). The N-terminal domain is required for interaction with cellular factors bound to mitotic chromosomes, such as the Brd4 protein (4, 59), and the C-terminal domain is required for binding to DNA sites in the viral genome (19, 52). Specific residues within the transactivation domain of E2 are required for interaction with Brd4 (4, 20, 49, 59), and the C-terminal dimerization function of E2 augments the E2-Brd4-mitotic chromosome interaction (5). We have previously shown that the domains required for chromosomal association of HPV8 E2 protein are different from those required for BPV1 E2 (44). Unlike BPV1 E2, the N-terminal domain of HPV8 E2 is not required for chromosomal association; instead, the C-terminal DNA binding and dimerization domain and the hinge region are essential. To date, no truncated E2 "repressor" proteins have been identified in the beta-HPVs, but there is a splice acceptor in the N-terminal region of HPV5 E2 hinge that could generate such a protein (17).

Should such proteins exist, we predict that they would be able to support maintenance and partitioning of the viral genome.

The betapapillomavirus E2 proteins have a unique intracellular localization in both interphase and mitotic cells. In interphase, the betapapillomavirus E2 proteins are observed in a nucleolus-excluded, fine granular pattern, throughout the nucleus, similar to the E2 proteins from other genera. However, the betapapillomavirus E2 proteins are additionally localized to nuclear speckles, which are enriched in splicing factors and are adjacent to sites of active transcription (25; this study). In mitosis, the beta-HPV E2 proteins associate with pericentromeric and rDNA loci on mitotic chromosomes. Figure 9 shows a model of cell cycle-specific localization of betapapillomavirus E2 proteins. In interphase cells, HPV8 E2 localizes to splicing speckles and transcription and replication foci but is excluded from the nucleoli. Because the rDNA genes are sequestered in the nucleoli in interphase, the E2 protein is free to participate in viral transcription and replication. When the nucleolar envelope breaks down in mitosis, E2 is able to interact with specific regions on the mitotic chromosomes. The number of
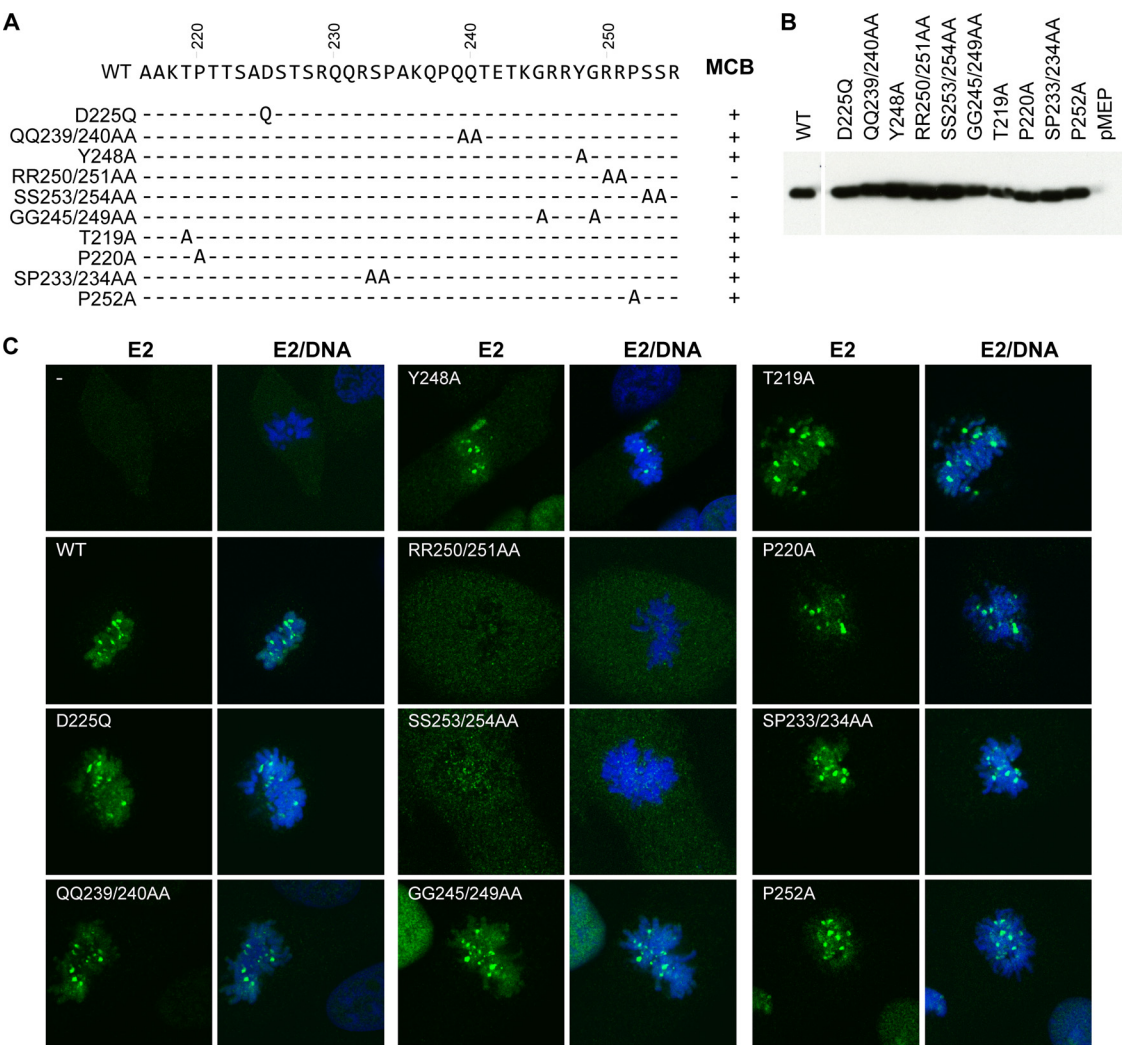
FIG. 6. Mutational analysis and mitotic chromosome binding phenotype of 216-255-C E2 proteins. (A) Amino acid substitutions in the background of E2 proteins with a 40-amino-acid region from the hinge (residues 216 to 255) fused to the CTD. The sequence from the wild-type (WT) E2 protein is shown at the top of the figure. The amino acids that are different from those in the WT protein are shown for the mutated E2 proteins. The mitotic chromosome binding phenotype (MCB) is shown to the right as follows: +, binds mitotic chromosomes; −, does not bind mitotic chromosomes. (B) Immunoblot analysis showing the expression levels of the mutated E2 proteins. E2 was detected using an anti-FLAG antibody. (C) Immunolocalization of the mutated 216-255-C E2 proteins in mitotic cells. Cells transfected with empty vector (−) are indicated. E2 proteins were detected using anti-FLAG antibody (green), and cellular DNA was stained with DAPI (blue). The percentage of positive E2-expressing mitotic cells (E2) showing the focus binding pattern (E2/DNA) is shown in Table 2.

TABLE 2. Percentage of E2-expressing mitotic cells showing the focus binding pattern seen in Fig. 6C

E2 protein	No. of mitotic cells examined	% Cells expressing E2	% E2-positive cells with E2 foci on mitotic chromosomes
WT (216-255-C)	33	69.7	91.3
D225Q	49	81.6	85.0
QQ239/240AA	50	92.0	95.7
Y248A	46	50.0	87.0
RR250/251AA	53	30.2	0
SS253/254AA	48	31.3	0
GG245/249AA	52	63.5	84.8
T219A	50	68.0	79.4
P220A	54	70.4	89.5
SP233/243AA	51	60.8	90.3
P252A	49	59.2	89.7

tandemly repeated rDNA units (400 per cell) will greatly increase the local concentration of the E2 binding target. RNA polymerase I and associated transcription factors remain bound to the rDNA loci in mitosis (36), and these regions also have specialized mechanisms for replication, cohesion, condensation, and segregation (8, 12, 24, 54, 56). The resulting, unique chromatin structure could provide unique targets for the betapapillomavirus E2 proteins.

In this study, we have mapped the regions of the HPV8 E2 protein required for localization to interphase splicing speckles. Lai et al. have shown that the HPV5 E2 protein is also associated with splicing factors and can augment splicing (25). In HPV8 E2, the hinge region is essential for nuclear speckle localization, but it is not sufficient; the C-terminal domain of E2 is also required for efficient nuclear speckle association.
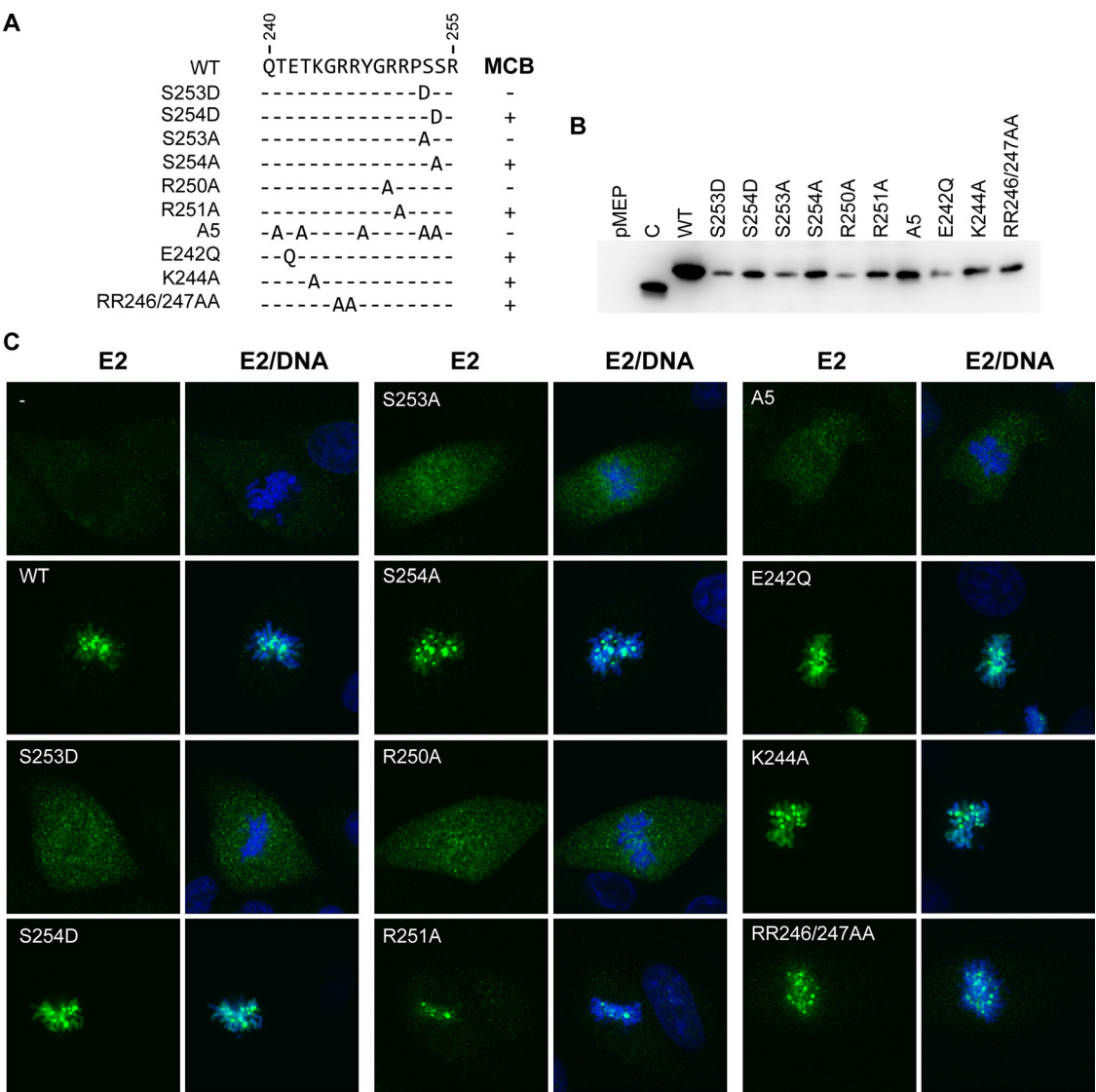
FIG. 7. Arginine 250 and serine 253 are critical residues mediating the chromosomal association function of the HPV8 E2 protein. (A) Amino acid substitutions in the background of E2 proteins with a 16-amino-acid hinge region (residues 240 to 255) fused to the CTD. The sequence from the wild-type (WT) E2 protein is shown at the top of the figure. The amino acids that are different from those in the WT protein are shown for the mutated E2 proteins. The mitotic chromosome binding phenotype (MCB) is shown to the right as follows: +, binds mitotic chromosomes; −, does not bind mitotic chromosomes. (B) Immunoblot analysis showing the expression levels of the mutated E2 proteins. E2 was detected using an anti-FLAG antibody. C, protein consisting of the CTD. (C) Immunolocalization of the mutated 240-255-C E2 proteins on mitotic chromosomes. Cells transfected with empty vector (−) are indicated. E2 proteins were detected using anti-FLAG antibody (green), and cellular DNA was stained with DAPI (blue). The percentage of E2-expressing mitotic cells and the percentage of positive E2-expressing mitotic cells showing the focus binding pattern are shown in Table 3.

Progressive removal of SR dipeptide motifs in the hinge region results in loss of nuclear speckle localization (Fig. 3B). However, expression of the hinge region alone results in nucleolar localization. In addition, proteins containing truncated regions of the hinge region fused to the C-terminal domain, which no longer associates with nuclear speckles, also localize to the nucleolus. Thus, in the presence of the DNA binding domain, nuclear speckle localization is dominant, but in the absence of this domain, E2 proteins derived from hinge and CTD are often observed in the nucleolus. It is not clear whether the nucleolar localization is of physiological significance. The hinge region contains many GR-rich regions that may function as

RNA binding regions that could associate with the large amounts of RNA found in the nucleolus. We have shown that the hinge region is important for the colocalization of HPV8 E2 with UBF and the ribosomal DNA loci on mitotic chromosomes (44), so localization to the nucleoli in interphase might not be unexpected. However, the mitotic chromosome binding region of the hinge (residues 240 to 255) does not localize E2 to the nucleolus. Furthermore, E2 proteins that do localize to the nucleoli are usually found in a diffuse pattern throughout the nucleolus and are not located specifically in the fibrillar centers or dense fibrillar components that contain the rDNA genes and UBF factor.

TABLE 3. Percentage of E2-expressing mitotic cells showing the focus binding pattern seen in Fig. 7C

E2 protein	No. of mitotic cells examined	% Cells expressing E2	% E2-positive cells with E2 foci on mitotic chromosomes
WT (240-255-C)	181	24.9	93.3
S253D	160	25	0
S254D	210	29	95.1
S253A	142	16.9	0
S254A	196	25	98.0
R250A	158	26.6	0
R251A	149	29.5	100
RR246/247AA	172	25.6	100
A5	180	23.9	0
E242Q	164	25.6	95.2
K244A	222	22.1	100

In this study, we have identified a 16-amino-acid chromosome binding region in the hinge that, when fused to CTD, is sufficient for E2 chromosomal association. Further analyses identified two specific residues within the mapped region, arginine 250 and serine 253, which are critical for the E2 mitotic chromosome binding function. Notably, the identified $R_{250}XXS_{253}$ motif, along with the arginine 250 and the serine 253 residues, is completely conserved among the 29 betapapillomaviruses that have been sequenced thus far. We have also demonstrated that the analogous chromosomal binding region in the HPV5 E2 protein confers an identical chromosomal binding phenotype. Thus, evolutionary conservation of this region in an otherwise divergent part of the E2 protein highlights its functional significance in the viral life cycle.

Arginine 250 and serine 253 lie within a consensus kinase motif, $R_{250}XXS_{253}$, and we have demonstrated that serine 253 is phosphorylated and that mutation of arginine 250 greatly decreases this modification. An overlapping $R_{251}XXS_{254}$ motif is not required for mitotic chromosomal foci, and serine 254 is

not a major phosphorylation site. However, mutation of arginine 251 also results in greatly reduced phosphorylation, although the resulting protein is not defective for chromosome binding. Therefore, an RRXS motif is required for phosphorylation, but only RXXS is required for localization to mitotic chromosomal foci. Thus, phosphorylation of serine 253 does not seem to be required for chromosomal binding of the 240-255-C E2 protein, unless this modification is cell cycle regulated and the residual phosphorylation observed is found primarily in mitotic cells. A second possibility is that the RXXS motif is directly required for mitotic chromosomal association but this interaction is blocked by phosphorylation. The model in Fig. 9 shows how E2 phosphorylation might directly regulate chromosome binding in mitosis. Another possibility is that phosphorylation might regulate another property of the E2 protein that is related to but not directly required for mitotic chromosome association. For example, the BPV1 E2 proteins are phosphorylated in the hinge region by CK2 and this modification triggers a conformational switch that targets the E2 protein for proteolysis through the ubiquitin-proteosome pathway. Thus, phosphorylation regulates the overall levels of the BPV1 E2 protein, which ultimately determines the number of genomes attached to mitotic chromosomes (32, 41, 42).

The RXXS motif is a consensus sequence for many protein kinases, including protein kinases A and C, calcium-calmodulin-dependent kinase, Aurora kinase B, and the ROCK kinases. Notably, some of these, such as Aurora kinase B, play an important role during mitosis in regulating host chromosomal segregation and cytokinesis. Phosphorylation of the E2 chromosome binding region requires more than the minimal $R_{250}XXS_{253}$ motif, since R251A results in decreased phosphorylation and the S254 in the overlapping $R_{251}XXS_{254}$ motif is not phosphorylated. The requirements for S253 phosphorylation indicate that the kinase recognition motif might be RRXS, a protein kinase A site.
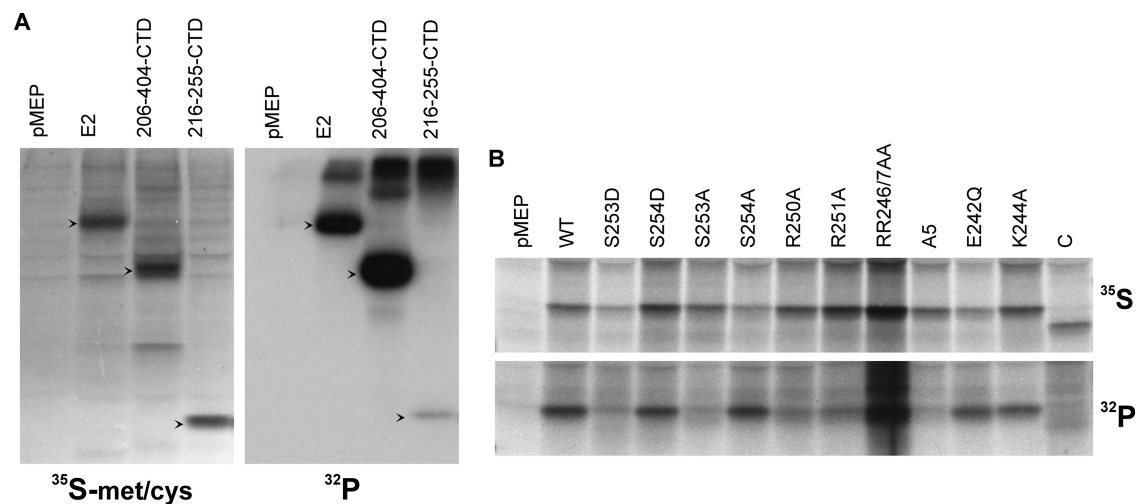

FIG. 8. Phosphorylation analysis of the HPV8 E2 proteins. (A) CV-1 cells expressing full-length E2 protein, HC protein (protein consisting only of the hinge and C-terminal domain [206-404-CTD]), and 216-255-C E2 proteins were metabolically labeled with either [^{32}P]orthophosphate or [^{35}S]methionine and cysteine. Equivalent amounts of total protein were immunoprecipitated using anti-FLAG beads, and the samples were analyzed by 15% SDS-PAGE. Incorporation of labeled isotopes was determined using a Phosphorimager. The positions of E2 proteins are indicated by black arrows on the gels. (B) CV-1 cells expressing the series of mutated proteins shown in Fig. 7A were metabolically labeled with [^{32}P]orthophosphate or [^{35}S]methionine and cysteine as described for panel A. WT, wild type 240-255-C E2 protein; C, CTD.
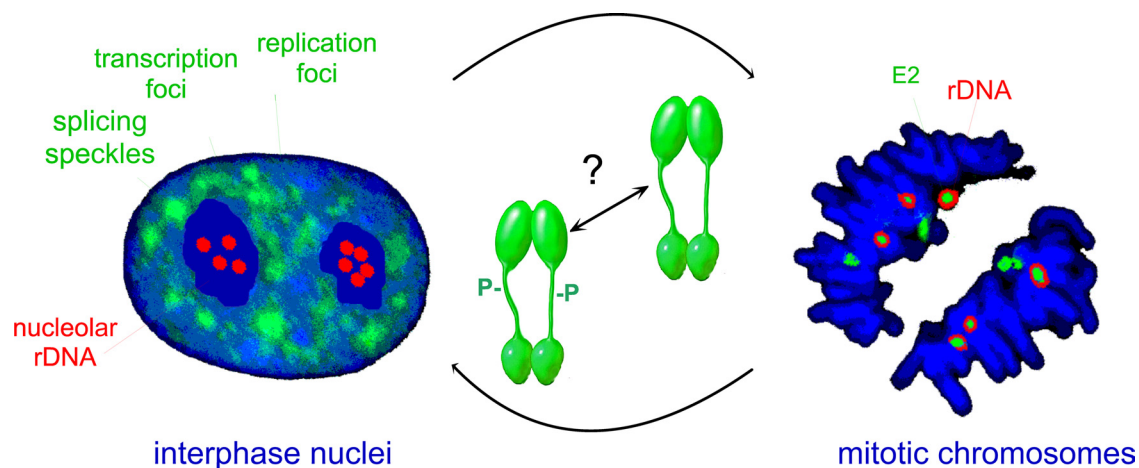
FIG. 9. Model of HPV8 E2 interactions at different stages of the cell cycle. In human interphase cells, HPV8 E2 localizes to splicing speckles and transcription and replication foci but is excluded from the nucleoli. When the nucleoli break down in mitosis, E2 is able to interact with specific regions on the mitotic chromosomes, such as the rDNA loci. Phosphorylation of the RXXS motif in E2 could modulate these interactions. For example, this motif might be required for interaction with the target on mitotic chromosomes but only in the unphosphorylated state. E2 is shown in green, rDNA is shown in red, and cellular chromatin is shown in blue. P, phosphate group.

Notably, the chromosome binding regions of LANA and EBNA have been mapped to short peptide sequences that have sequence similarities to the chromosome binding region of HPV8 E2 that we have mapped in this study. As shown in Fig. 10, the identified RXXS kinase motif is also present in the chromosome binding region of the LANA and EBNA1 tethering proteins. In EBNA1, several of the RXXS motifs overlap with AT hook regions that are required for chromosome binding and partitioning (47). In LANA, substitution of residues overlapping the RXXS motif also disrupts episomal persistence, chromosome binding, and interaction with histones H2A and H2B (2). In addition, the chromosome binding regions all contain GR motifs. In EBNA1, these arginine residues are methylated (51), the serine residues are phosphorylated, and substitution of the serines disrupts the EBNA partitioning function (10, 51). EBNA1 is thought to associate with mitotic chromosomes by either interacting directly with chromosomal DNA through AT-hook DNA binding regions (47) and/or or by interacting with a host nucleolar protein human EBP2 (7, 50). These observations suggest that, although these proteins may have different chromosomal targets, phosphorylation by certain cell cycle-dependent kinases could be a common mechanism of regulation of chromosome binding function.

The C-terminal domain of HPV8 E2 is required for mitotic chromosome association and localization to nuclear speckles. This domain mediates dimer formation (31) and specifically binds to a DNA motif that is found at multiple positions in the viral genome (1). Either the DNA binding or dimerization function could be required for interaction with mitotic chromosomes. In fact, we have found that the C-terminal dimerization function is required for efficient association of BPV1 E2 with Brd4 and with mitotic chromosomes (5). Another possibility is that the C-terminal domain is required for direct association with cellular proteins on mitotic chromosomes and that this interaction cooperates with the binding or regulatory role of the RXXS motif. There is precedence for this association in studies of the LANA C-terminal domain; mutations that do not disrupt DNA binding or dimerization of LANA can abrogate mitotic chromosome binding (23). Notably, the LANA C-terminal region also localizes to pericentromeric regions of mitotic chromosomes (22), suggesting that LANA and E2 might have a common target.

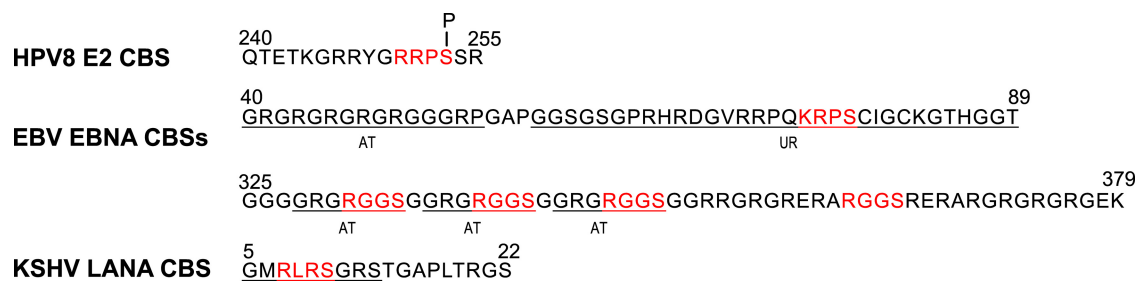Future studies will address the molecular interactions of the

FIG. 10. Similarities among the chromosome binding regions (CBSs) of HPV8 E2, EBNA1, and LANA. AT hook regions are underlined, and RXXS motifs are shown in red. The E2 serine 253 residue, shown to be phosphorylated in this study, is indicated by a P above the S. The EBNA1 "unique region," (UR), important for transactivation, is also indicated. EBV, Epstein-Barr virus; KSHV, Kaposi's sarcoma-associated herpesvirus.

chromosome binding region of the betapapillomavirus E2 proteins. Several functions and protein interactions have already been assigned to the PV E2 hinge region. For example, the HPV8 E2 hinge region can activate transcription by recruitment of Sp1 to cellular promoters (53). The studies presented here show that the full-length HPV8 E2 protein interacts with several different complexes that direct its intracellular localization. One of the strongest associations is with nuclear speckles. As mentioned above, the hinge of HPV5 E2 interacts with splicing factors and enhances splicing. The N-terminal domain of HPV8 E2 binds the Brd4 protein, and this interaction strengthens the association of Brd4 with cellular chromatin (35). However, Brd4 is not required for the prominent foci observed bound to the rDNA loci on mitotic chromosomes (44). In the absence of the N-terminal domain and the RS regions of the hinge, the E2 protein was observed in the nucleolus. As discussed above, this localization might be due to putative RNA binding regions of the hinge region or might be due to interaction with nucleolar proteins.

The betapapillomavirus E2 proteins have properties distinct from those of E2 proteins from other papillomavirus genera. To date, interphase localization to nuclear speckles and mitotic association with rDNA loci has been characterized only for the beta-HPV E2 proteins. HPV8 E2 can give rise to tumors in transgenic mice (43), and it will be of interest to see whether any of these unique properties are connected and relate to the unique pathogenesis of beta-HPV infection. The experiments described here have characterized sequences required for the interaction of the HPV8 E2 protein with mitotic chromosomes. By analogy with other papillomaviruses, we assume that this interaction is crucial for the virus to tether its genome to host chromosomes during persistent infection. Understanding the mechanism by which the E2 protein tethers the viral genome to host chromosomes during persistent infection will assist in the development of antiviral therapies to inhibit E2 interaction with mitotic chromosomes and thus eliminate viral genomes from infected cells.

ACKNOWLEDGMENTS

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